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mtDNA Ribosomal Gene Phylogeny of Sea Hares in the Genus *Aplysia* (Gastropoda, Opisthobranchia, Anaspidea): Implications for Comparative Neurobiology

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Abstract.—Sea hares within the genus *Aplysia* are important neurobiological model organisms; as more studies based on different *Aplysia* species are appearing in the literature, a phylogenetic framework has become essential. We present a phylogenetic hypothesis for this genus, based on portions of two mitochondrial genes (12S and 16S). In addition, we reconstruct the evolution of several behavioral characters of interest to neurobiologists to illustrate the potential benefits of a phylogeny for the genus *Aplysia*. These benefits include determination of ancestral traits, direction and timing of evolution of characters, prediction of the distribution of traits, and identification of cases of independent acquisition of traits within lineages. This last benefit may prove especially useful in understanding the linkage between behaviors and their underlying neurological bases. [Anaspidea; *Aplysia* phylogeny; neurobiology of inking; neurobiology of swimming; Opisthobranchia; sea hare.]

The nervous system of sea hares in the genus *Aplysia* is relatively simple and easily manipulated experimentally, being composed of ~20,000 giant nerve cells. This has made species in this genus popular model organisms for examining the molecular and cellular basis of basic neural processes common to invertebrates and vertebrates (Kandel, 1979; Carew, 1987). Numerous investigations of the behavioral and neurological traits of *Aplysia* species have been published, primarily focusing on *Aplysia californica* and, to a lesser extent, *A. brasiliana*, *A. dactylomela*, *A. depilans*, *A. fasciata*, *A. juliana*, *A. kurodai*, *A. punctata*, and *A. parvula*. Unfortunately, a phylogenetic framework is lacking, although some authors (e.g., Kandel, 1979) have emphasized the need for an insight into the relationships of *Aplysia* species to be able to understand the evolution of behavioral traits and their underlying neural processes.

A phylogenetic framework permits a more rigorous testing of the evolution of the behavioral mechanisms studied by neurobiologists. The power of this approach is exemplified in two recent studies of neurological trait evolution within the Anaspidea (see below). Wright et al. (1996) utilized a morphology-based phylogeny of the Anaspidea to demonstrate that the genus *Dolabrifera* has lost two well-studied neuromodulatory learning mechanisms: serotonin-induced increase in spike duration, and excitability of tail sensory neurons. On the basis of molecular evidence suggesting that *Dolabrifera* and *Phyllaplysia* are sister taxa (Medina, 1998; Medina and Walsh, 2000), Erixon et al. (1999) tested several evolutionary hypotheses at both cellular and behavioral levels. In *Aplysia*, the presence of both neuromodulatory traits is associated with the presence of the sensitization phenotype. In *Dolabrifera*, the absence of both neuromodulatory traits is associated with the absence of sensitization (Wright, 1998). This correlation between neural and behavioral phenotypes appears to break down in *Phyllaplysia*, however, which shows the neuromodulatory traits but no sensitization. Erixon et al. (1999) concluded that at the neuromodulatory level *Phyllaplysia* resembles ancestral sea hares (i.e., *Aplysia*), whereas at the behavioral level they resemble their sister taxon *Dolabrifera*. These studies addressed evolution at the level of families within the order. Comparative work has also been carried out within the genus *Aplysia*. Different species of *Aplysia* exhibit variations in behavioral traits such as nocturnal or diurnal activity (Carefoot and Thomas, 1992), swimming, crawling (Eales, 1960; Kandel, 1979), burrowing (Carefoot, 1987), and inking behavior (purple or white) as responses to predation or other environmental stimuli (Nolen et al.,

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1995; Johnson and Willows, 1999). The neurological pathways for inking (Carew and Kandel, 1977a, b, c) and swimming behavior have been identified for different species (Von der Porten and Parsons, 1982; Gamkrelidze et al., 1995), but the absence of a phylogenetic hypothesis for *Aplysia* species has prevented an investigation of the timing, causes, correlated changes, and frequency of evolution of these traits.

We produce here a phylogenetic hypothesis for the genus *Aplysia* based on new molecular data from the present study and compare this hypothesis with previous classifications. In addition we illustrate the utility of this phylogeny by mapping several published behavioral adaptations (swimming, inking, and siphon and gill withdrawal) onto this phylogeny and inferring the likely evolutionary histories of these traits.

Materials and Methods

Classification

Aplysia is a genus within the family Aplysiidae, united with the Akeridae in the suborder Anaspidea, which is in turn nested within the cephalaspid opisthobranchs or bubble shells (Mikkelsen, 1996; Medina and Walsh, 2000). *Aplysia* species have been separated into five subgenera based on morphology (Eales, 1960), although no data matrix or modern systematic treatment supports these divisions. *Pruvotaplysia* Engel 1936 has been considered the most basal subgenus, followed by the subgenus *Aplysia* Linnaeus 1767 (Eales, 1960). *Varria* Eales 1960 is the most species-rich subgenus. The subgenera *Neaplysia* Cooper 1863 and *Phycophilia* Engel 1936 are monotypic. Approximately 35 species of *Aplysia* have been described, 9 of which have been used for neurological/behavioral studies. A total of 15 taxa were included in the study: the outgroup *Akera*, sister taxon to the Aplysiidae (Gosliner, 1991, 1994; Mikkelsen, 1996; Wright et al., 1996; Medina and Walsh, 2000), and 14 *Aplysia* species representing four subgenera (Table 1). The rarity and pelagic existence of the subgenus *Phycophilia* prevented us from obtaining representatives of this taxon.

Genes Analyzed

Portions of the small (12S) and large (16S) mitochondrial ribosomal genes were sequenced for phylogenetic analysis. Estimates of the maximum time-scale for reliable application of these genes in phylogenetic reconstruction vary widely by taxon and among studies, ranging from 65 million years ago (mya) (Hillis and Dixon, 1991) through 100 mya (Ortí and Meyer, 1997) to 300 mya (Mindell and Honeycutt, 1990). The time of the origin of the Aplysiidae has been bracketed by both fossil and molecular data. The earliest occurrence of the genus *Akera*, an anaspidean taxon with a strong bulloid shell, and the sister taxon to the Aplysiidae, is 165 mya (Tracey et al., 1993). Both shell size and shell calcification are reduced in *Aplysia*, making it a poor candidate for fossilization. *Aplysia* has a limited fossil record, dating to Burdigalian Age Miocene deposits (Tracey et al., 1993). The fossil record therefore suggests that the divergence of *Aplysia* species began between 165 and 20 mya. A comparative study of the egg-laying hormone gene estimated the divergence of the common ancestor of *A. parvula* (a primitive sea hare) and *A. californica* to be 140 mya (Nambu and Scheller, 1986). This date was based on a calibration of the divergence of *Aplysia* and *Lymnaea*, estimated at 350 mya. Given the small size (35 amino acids), high level of sequence divergence between the *Aplysia* and *Lymnaea* egg-laying hormone genes (>55%), and the failure to correct for multiple substitutions and invariant sites, the age of divergence of *Aplysia* species probably has been overestimated. Thus, fossil and molecular data suggest that *Aplysia* species diverged between 165 and 20 mya—probably closer to the younger end of this range, and well within the overall range of estimates of the utility of these genes.

DNA Isolation, PCR Amplification, and Phylogenetic Methods

Total DNA was isolated by standard sodium dodecyl sulfate/Proteinase K digestion (Sambrook et al., 1989), and resuspended in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). Amplification and sequencing were performed according to the protocol described in Medina and Walsh (2000). A region of ~430 bp of the 16S ribosomal gene was sequenced by using the primers 16Sar-L (5′-CGCCTGTTTA TCAAAAACAT-3′) and 16Sbr-H (5′-CCG-GTCTGAACTCAGATCACGT-3′) (Palumbi et al., 1991). A region of ~367 bp was

TABLE 1. Species examined in this study, locality, and collector.

Species	Locality	Collector
Akera bullata	Algoleran, Sweden	J. M. Turbeville
Aplysia		
Pruvotaplysia		
A. parvula	Kapalua Bay, Hawaii	Cory Pittman
A. punctata	Asturias, Spain	Jesús Ortea
Aplysia		
A. depilans	Mediterranean Sea, Israel	Avy Susswein
A. juliana	Habana, Cuba	Jesús Ortea
A. vaccaria	San Diego, California	Lisa Angeloni
Neaplysia		
A. californica	Mission Bay, California	Mónica Medina
Varria		
A. brasiliana	Biscayne Bay, Florida	Tom Capo
A. cervina	Gulf of Mexico, Texas	Ned Strenth
A. dactylomela	Florida Bay, Florida	Tom Capo
A. fasciata	Mediterranean Sea, Israel	Avy Susswein
A. gigantea	Hope Town, Western Australia	Clay Bryce
A. kurodai	Kobe, Japan	Tatsumi Nagahama
A. morio	San Padre Island, Texas	Ned Strenth
A. oculifera	Rottnest Islands, Western Australia	Clay Bryce

sequenced from the 12S gene by using the primers 12Sa-L (5′-AAACTGGGATTAGATACCCCACTAT-3′) and 12Sb-H (5′-GAGGGTGACGGGCGGTGTGT-3′) (Palumbi et al., 1991). The sequence data reported here have been deposited in GenBank under the accession numbers AF156111-113, AF156127-129, and AF192279-302. For text: "Six 12S and 16S sequences were previously used by Medina and Walsh (2000), for *Akera bullata, A. cervina* and *A. punctata* AF156111-113, AF156127-129. The remaining sequences were generated for this study (AF192279-302)". The 16S and 12S sequences were aligned by using the default settings of Clustal W (Thompson, 1994) (alignment available on the *Systematic Biology* website, http://www.utexas.edu/ftp/depts/systbiol/). To improve the comp- uter-generated alignment, we developed secondary structure models for the genus *Aplysia* (Medina and Walsh, 2000). The alignment for 16S required 4–8 indels (insertion/deletion events) per sequence (1.2–2.2% of the aligned 16S sequence length). Most gaps were one nucleotide (1 nt) long, but a few indels were as large as 4 nt. The alignment for 12S required 5–9 indels per sequence (1.6–2.9% of the aligned 12S sequence length), and again, most gaps were 1 nt long; a few indels, however, took up as much as 5 nt, and one gap of 17 nt was found in helix 31 of the outgroup *Akera*.

The ILD test (Farris et al., 1995) implemented in PAUP* 4.0 was used to determine whether the two gene regions possessed significantly conflicting phylogenetic signal. The test settings were 10 random stepwise additions with TBR branch swapping and 1,000 randomizations. All phylogenetic analyses were conducted in PAUP* 4.0 (Swofford, 2000). Modeltest version 2.0 (Posada and Crandall, 1998) was used to determine which model of sequence evolution best fit the data, based on nested likelihood ratio tests (LRT). Maximum likelihood (ML) trees were produced from 100 random additions in PAUP* 4.0 with TBR branch swapping. For maximum parsimony (MP) analysis, gaps were treated as missing characters. A heuristic search was performed, with 100 replicates of random stepwise addition and TBR branch swapping. Bootstrap analysis with 1,000 replicates (Felsenstein, 1985) in PAUP* 4.0 (1 and 10 random additions for ML and MP, respectively) and Decay analysis in TreeRot (Sorenson, 1996) were conducted to estimate branch support. Ancestral character state reconstruction was performed by using a MP approach in MacClade 3.06 for three characters linked to behavior (Maddison and Maddison, 1992). In the case of swimming, in order to interpret the equivocal reconstructions for certain nodes, branches with a bootstrap support <70% were collapsed and subsequently randomly resolved 1,000 times. Finally, we obtained an average estimate for how many times swimming evolved.

RESULTS

Secondary Structure Models and Alignment

The secondary structure model for 16S is presented in Figure 1. Loop regions are identified according to the numbering of Horovitz and Meyer (1995). A sliding window analysis (7 nt) similar to the one presented previously (Medina and Walsh, 2000) was used to identify variable regions. The most variable regions, identified by lowercase letters in Figure 1, were found in loops.

The structural model of the 12S third domain of *Aplysia cervina* is presented in

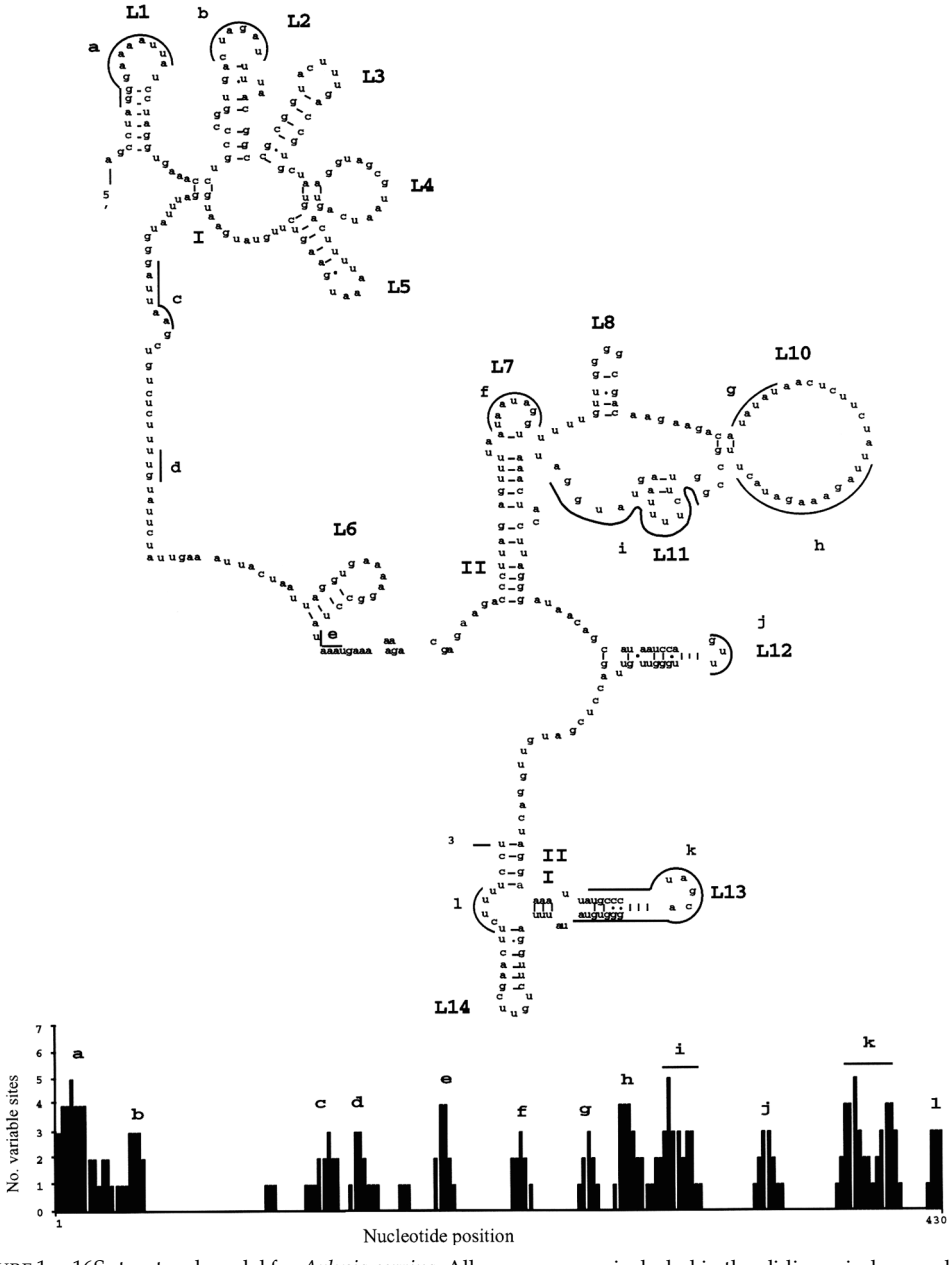

FIGURE 1. 16S structural model for *Aplysia cervina*. All sequences are included in the sliding window analysis of the 16S molecule at the bottom. The vertical axis represents the number of variable sites in a sliding window of 7 nt. The horizontal axis represents the position in the multiple alignment. Lowercase letters ($a - l$) indicate the most variable regions in the structural model.

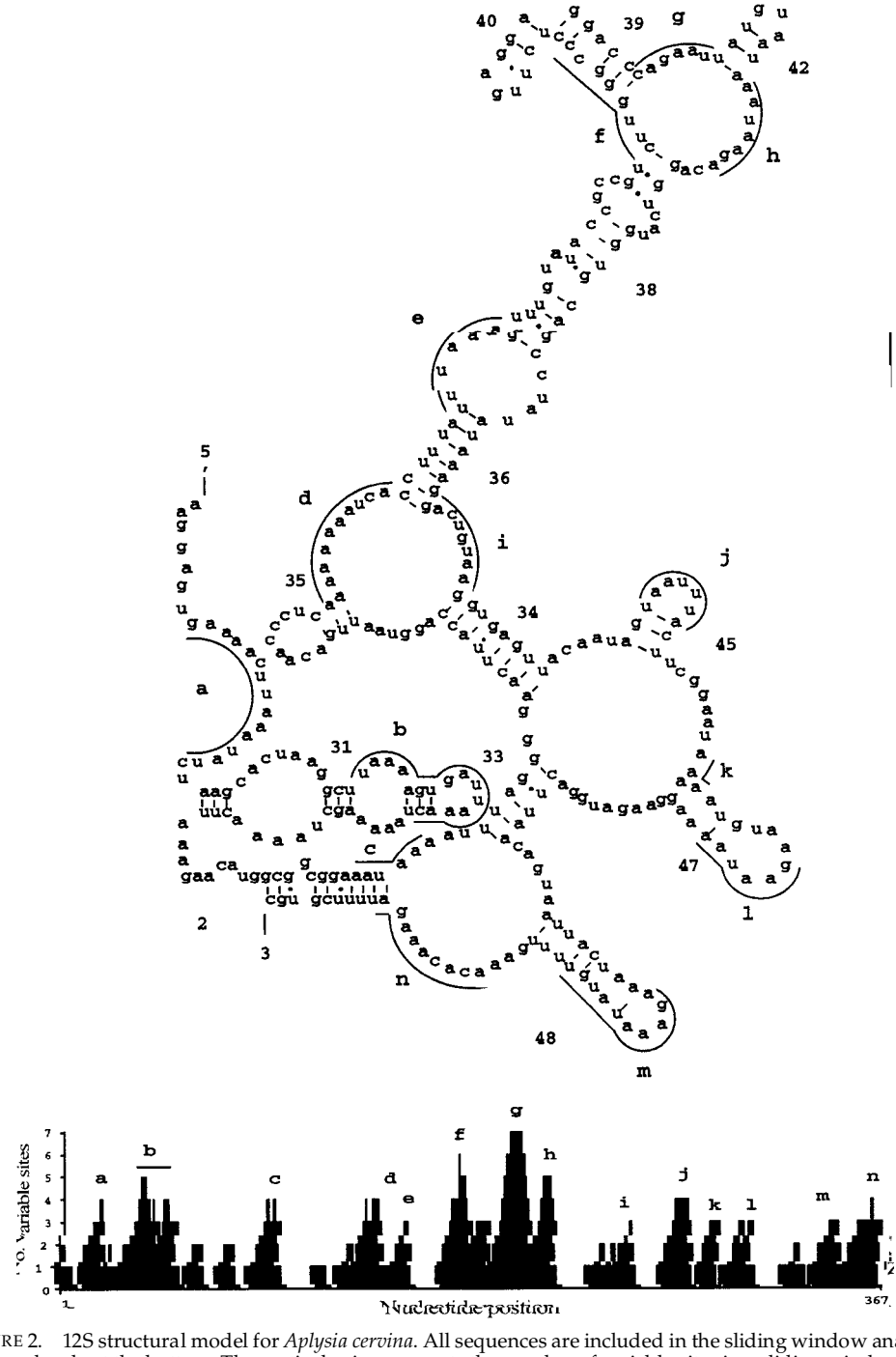

FIGURE 2. 12S structural model for *Aplysia cervina*. All sequences are included in the sliding window analysis of the 12S molecule at the bottom. The vertical axis represents the number of variable sites in a sliding window of 7 nt. The horizontal axis represents the position in the multiple alignment. Lowercase letters ($a - n$) indicate the most variable regions in the structural model.

Figure 2, with the variable regions highlighted. Helices were numbered according to Van de Peer et al. (1994). In the loop of helix 31, all *Aplysia* sequences had an insertion of variable size (13–17 bp) that is absent in the outgroup. This insert appears to be a synapomorphy for the genus *Aplysia*. No other anaspidean taxon sampled

(Medina and Walsh, 2000) possessed this expanded loop in helix 31 (anaspidean secondary structures are available from the NIH-*Aplysia* Resource Facility website, http://www.rsmas.miami.edu/groups/seahares/).

An initial alignment was generated with Clustal W (Thompson, 1994) using the default settings. Problematic areas of alignment were primarily within loop regions. Encoding secondary structure did, however, improve the alignment in stem regions (Fig. 3). For instance, Clustal W did not properly align stem regions 31.c (see legend to Fig. 3 for nomenclature) and 31.c' because of length variation in helix 31 of the 12S gene. Consideration of secondary structure improved this part of the alignment sufficiently that these characters could be included with confidence in the final analysis. A simpler example can be observed in helix 2 of 12S, where stem regions were clearly identified and the alignment program placed three gaps in the stem region 2' rather than in the unpaired region of *Aplysia juliana*. These gaps were deletions in the structural region identified by *n* in Figure 2.

Molecular Evolution

The 16S alignment consisted of 430 positions, of which 71 were variable and 26 were parsimony informative. The 12S alignment consisted of 367 positions, of which 97 were variable and 39 were parsimony informative. The average nucleotide composition for 16S was 30% A, 33% T, 15% C, and 22% G. The average nucleotide composition for 12S

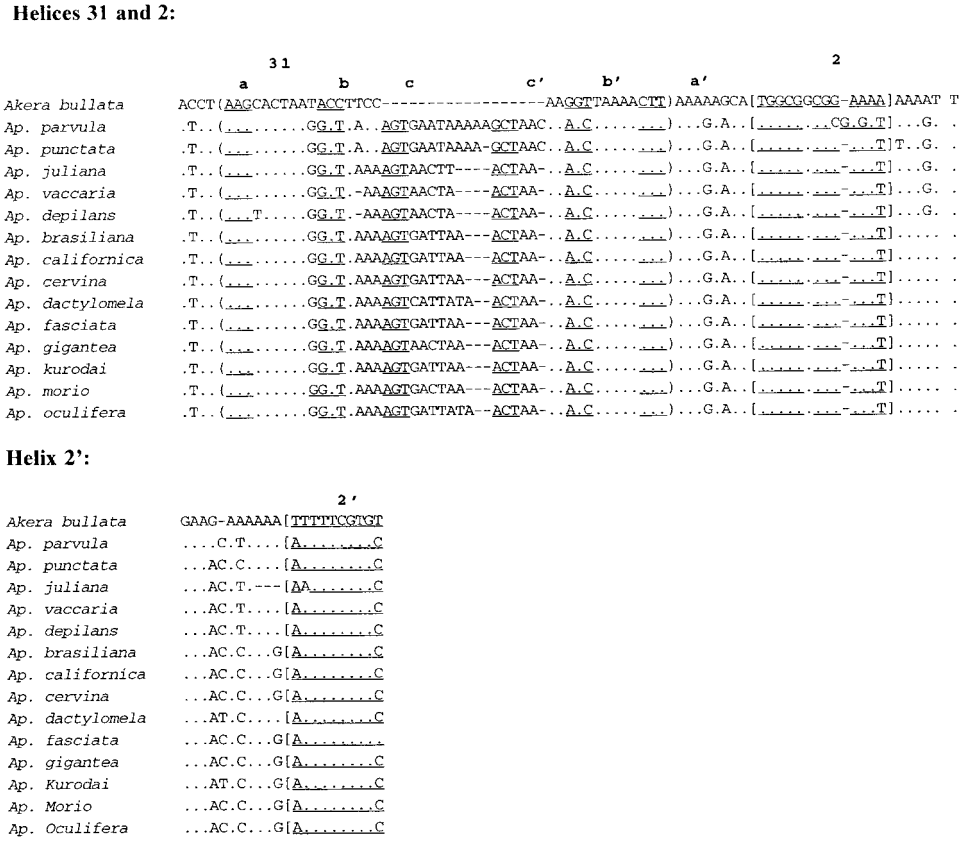

FIGURE 3. Partial alignment of the 12S gene. Secondary structure is indicated in the alignment according to the nomenclature proposed by Kjer (1995), in which parentheses indicate helices (i.e., helix 31) and the underlined regions indicate the nucleotides involved in stem pairings. Regions in brackets (i.e., helix 2) represent half stems that are separated by other structural domains, and again the underlined regions represent nucleotides involved in pairings. The corresponding half-stem is represented by the same number. The numbers for structural domains follow the helices numbering of Van de Peer et al. (1994) for 12S.

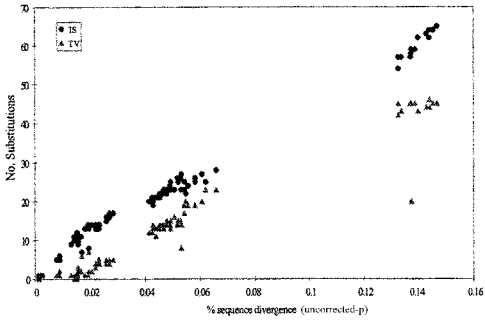

FIGURE 4. Plot of observed number of substitutions, (transitions [Ts] and transversions [Tv]) versus percent sequence difference in the two rDNA gene fragments sequenced for all taxa, including the outgroup.

was 40% A, 25.5% T, 14.5% C, and 20% G. Sequence differences within *Aplysia* ranged from a single substitutional difference between *A. brasiliana* and *A. fasciata* to 7.6% uncorrected distance between *A. parvula* and *A. juliana*. The outgroup sequence differed by 13–15% from the *Aplysia* sequences. All species differed by ~1% from the averages, except for the outgroup, which differed by ~2.5% from the averages. The ILD test indicated that the two ribosomal genes (16S and 12S) did not have significantly conflicting phylogenetic signals ($P = 0.299$); therefore, they were combined for all subsequent analyses. Observed transitions and transversions were plotted versus percent sequence difference for all comparisons (Fig. 4). The plotted observations indicated that transitions continue to outnumber transversions, even in the comparisons between the ingroup and the outgroup, suggesting that transitions are not fully saturated. The model that best fitted the data was the HKY85 (Hasegawa et al., 1985) with a transition/transversion ratio of 3.433 : 1, a proportion of invariant sites of 0.547, and gamma = 0.423. This model was used for the ML analysis.

Phylogeny

The three best trees recovered when using the ML criterion ($-\ln L = 2341.885$) differed solely in the placement of *Pruvotaplysia* species. In one tree *A. parvula* and *A. punctata* appear as a clade that is the sister taxon to the rest of the *Aplysia* clade (Fig. 5); in the second tree they form a trichotomy with the *Aplysia* clade (subgenus *Aplysia*, not shown); and in the third tree (not shown) they are sequential sister taxa to the *Aplysia* clade (subgenus *Aplysia*), with *A. parvula* branching first. Two other distinct clades were present in the ML trees, the subgenus *Aplysia* clade and the subgenus *Varria* clade, with *A. californica* (subgenus *Neaplysia*) as part of the *Varria* clade. The bootstrap support for the MP and ML analyses is presented in Figure 5.

An equal-weights MP analysis produced seven shortest trees of 261 steps with a consistency index of 0.751. The strict consensus tree of the seven MP trees does not support monophyly of the subgenus *Pruvotaplysia*, even though both of its members (*A. parvula*

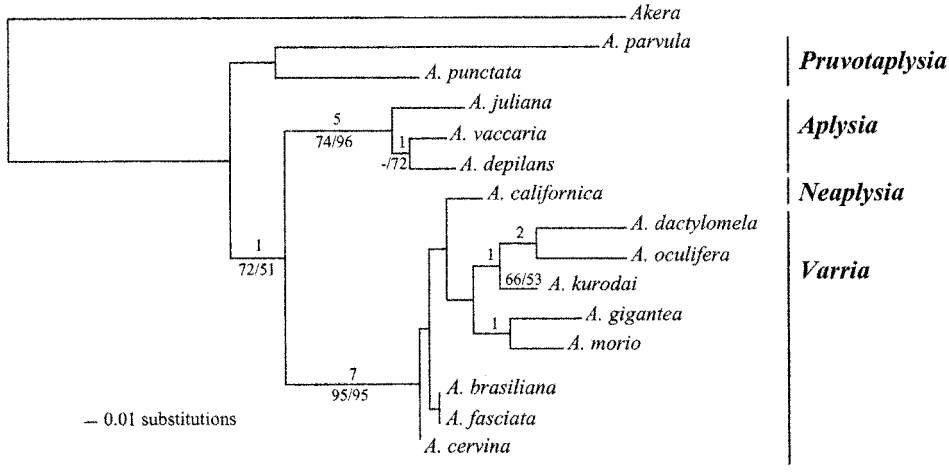

FIGURE 5. One of three ML trees found with the combined 12S and 16S dataset for the genus *Aplysia*; *Akera* is used as the outgroup. Decay values are depicted above branches and bootstrap values (ML/MP) below. Eales's (1960) subgenera are listed in bold on the right.

and *A. punctata*) appear as the most basal taxa in the tree (tree not shown). Some of the ML clades within the subgenus *Varria* have been lost, but both the *Aplysia* and *Varria* subgenera are monophyletic in the consensus with strong bootstrap support (Fig. 5). In this analysis the subgenus *Neaplysia* appears again as part of the *Varria* clade.

Discussion

Aplysia Phylogeny

The alignment approach used in this study indicates that secondary structure encoding allowed the confident inclusion of more characters. Despite the modest sequence divergences reported, the rRNA gene regions analyzed contained sufficient signal to resolve some divergences in the *Aplysia* phylogeny with good support (Fig. 5).

The only modern attempt to reconstruct phylogenetic relationships between *Aplysia* species was based on myoglobin amino acid data for just three taxa (Rinaldi and Ophir, 1998). The results suggested that the subgenus *Varria* (consisting of two species—*A. fasciata* and *A. kurodai*) was a paraphyletic group ancestral to *A. juliana* (subgenus *Aplysia).* The molecular phylogeny we present is largely congruent with the subgeneric apportionment of Eales (1960), supporting monophyly of the subgenera *Aplysia* and *Varria*. The only clear incongruence between the molecular evidence presented here and Eales's subgeneric classification is the placement of *A. californica.* Molecular evidence nests the subgenus *Neaplysia* within the *Varria* clade. Because the subgenus *Varria* contains the more recent divergences within the *Aplysia* clade, the ribosomal genes lack sufficient resolution at this level. Mitochondrial cytochrome oxidase 1 sequences have been used to resolve relationships within this subgenus (Medina, 1998; Medina et al., in prep). Preliminary results support the nesting of the subgenus *Neaplysia* within *Varria*. Our results with the rRNA genes also support previously published speculations concerning the phylogenetic position of the subgenus *Pruvotaplysia* (Eales, 1960). The species belonging to the subgenus *Pruvotaplysia* (*A. parvula* and *A. punctata*) were suggested as the most basal taxa in the genus *Aplysia*.

Evolution of Cerebralization

Given the phylogeny presented above, and knowledge of the distribution of traits in living taxa, one can draw reasonable inferences concerning the direction, timing, and number of times that traits have arisen. For instance, character polarity, the determination of the ancestral state of a trait or character, may have important implications for the evolution of traits. An example of character polarity determination can be seen in the cerebralization of the nervous system, a process that involves fusion of the nervous ganglia (Gosliner, 1994). Primitive opisthobranchs have long lateral nerve cords and distinct ganglia; as cerebralization occurs as an evolutionary progression, the nerve ganglia fuse and the lateral cords are shortened. In the case of *Aplysia*, the nervous system of the subgenus *Pruvotaplysia* species resembles that of the outgroup *Akera*, whereas the more derived subgenera possess fused ganglia (Wright, 1975; Fig. 6).

Evolution of Swimming

Homoplasy, expressed as reversals, parallel evolution, or convergence, can be misleading in phylogenetic studies. It can, however, be informative about character evolution when properly identified within a phylogenetic context. The repeated evolution of similar traits presents an opportunity to discover similar patterns of character evolution and factors shaping the evolution of these traits. These repeated "experiments" in character evolution may prove much more tractable than singular events in understanding evolutionary processes and the linkage between behaviors and underlying neurological mechanisms. An example of this is the evolution of swimming behavior; a trait that has been lost and gained in several lineages.

Although swimming appears to be the ancestral condition in the Anaspidea (Medina and Walsh, 2000), this behavior evolved several times in the genus *Aplysia*. Members of the subgenus *Pruvotaplysia* are the only sea hares that lack the ability to swim, whereas members of the *Aplysia* and *Varria* clades do exhibit swimming behavior. On average, this character seems to have evolved between 4.4 and 6 times, regardless of the alternative resolution of basal clades (Fig. 7). Occasional swimming events have been reported from

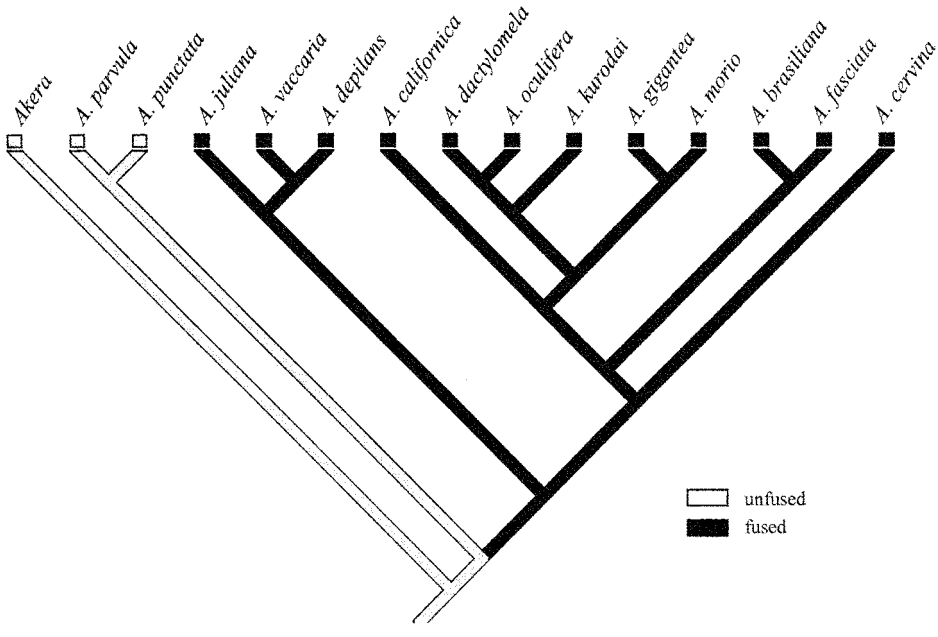

FIGURE 6. Evolution of cerebralization in the genus *Aplysia*. Fusion and shortening of lateral nerve ganglia is a common trend in derived opisthobranch gastropods. The outgroup *Akera* and the species in the subgenus *Pruvotaplysia* (the most ancestral members of the genus) exhibit a primitive nerve system with longer lateral nerve cords and less ganglia fusion. Character optimization was performed by MP in Maclade 3.06 (Maddison and Maddison, 1992).

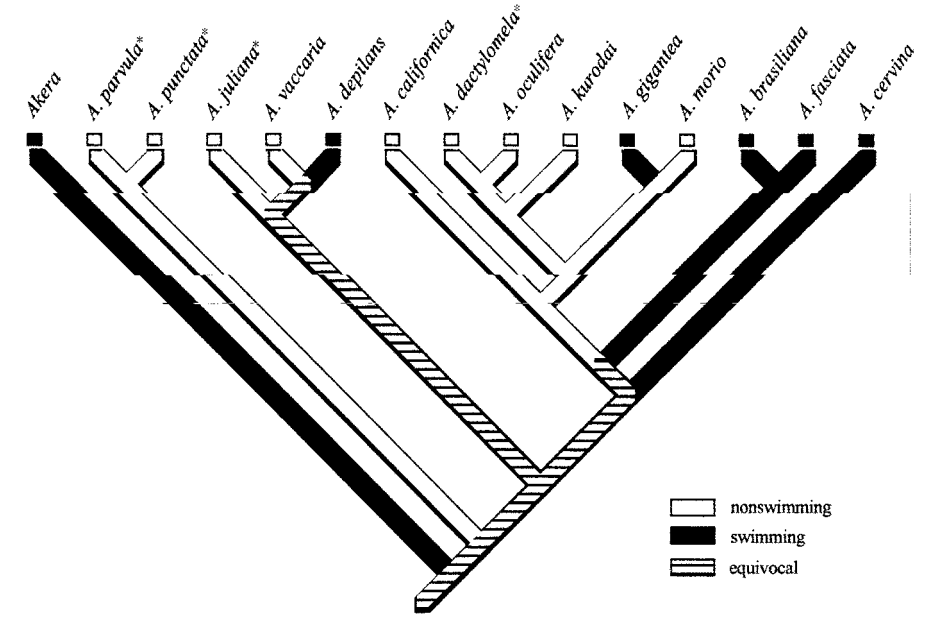

FIGURE 7. Swimming behavior mapped onto the topology of the molecular data. Swimming seems to be a plastic behavior that evolved multiple times in the genus *Aplysia*. *Occasional swimmers have been reported in the literature (see review in Johnson and Willows, 1999). Character tracing was performed by MP in MacClade 3.06 (Maddison and Maddison, 1992).

species considered to be nonswimmers (summarized in Carefoot, 1987; Johnson and Willows, 1999). Johnson and Willows (1999) hypothesize that *Aplysia* species could swim if they possessed sufficiently large parapodia, a possibility that explains the occasional reports of unexpected swimming in certain species.

The neural circuit that induces swimming in *A. brasiliana* has been identified; it involves neurons located in the cerebral ganglion (Gamkrelidze et al., 1995) and the two pedal ganglia (Von der Porten and Parsons, 1982). Gamkrelidze et al. (1995) concluded that most of the same cells are also involved in controlling crawling behavior in *A. californica.* Consequently, the neural pathway that controls movement in *Aplysia* species appears to be an acutely plastic mechanism. We hypothesize that either crawling or swimming behavior was adopted as a response to the environmental challenges each species faced. Several lines of evidence support modulatory models for the neurons and parapodial muscle fibers involved in swimming behavior (Gamkrelidze et al., 1995; Laurenti and Blankenship, 1996, 1997). The availability of a relatively accurate map of the neural circuit on *A. brasiliana* and the understanding of the neurophysiology of swimming in this species will facilitate the assessment of homology of neural circuits among other species. The phylogenetic hypothesis presented here, with a more thorough study of other *Aplysia* species, will allow identification of the similarities and differences in neural mechanisms utilized during independent cases of the evolution of swimming in this genus.

Evolution of Inking

The release of white or purple ink in response to a noxious stimulus is controlled by a small group of neurons in the abdominal ganglion (Carew and Kandel, 1977a, b, c; Byrne, 1980). Two glands in sea hares produce an external secretion, the opaline gland (located beneath the floor of the mantle cavity) and the ink or purple gland (located near the mantle edge, over the gill). Inking behavior is widespread throughout the genus *Aplysia*, except for the subgenus *Aplysia*, which releases a white secretion from the ink gland (Marcus and Marcus, 1955; but see review in Johnson and Willows, 1999); indeed, this characteristic was used to diagnose the subgenus (Eales, 1960; Engel and Eales, 1957). The molecular evidence strongly supports monophyly of the subgenus *Aplysia*, which, in turn, suggests that white secretions from the ink gland are a synapomorphy of this clade (Fig. 8).

Several hypotheses concerning the function of inking behavior in the genus *Aplysia* have been proposed, including camouflage, predator deterrence, reproductive isolation between coexisting species, and waste excretion (Kandel, 1979; Carefoot, 1987; Carefoot et al., 1999; Johnson and Willows, 1999). Recent reviews conclude that *Aplysia* inking behavior probably evolved as a defense mechanism to deter natural predators (Nolen et al., 1995; Johnson and Willows, 1999).

If purple inking is a defense mechanism, then species from the subgenus *Aplysia*, which have lost this predator deterrent behavior, may be more vulnerable to predation. The lack of purple ink may therefore have resulted in the need for alternative antipredator mechanisms. The secretion of the opaline gland, like ink release, is correlated with noxious stimuli (Tritt and Byrne, 1980). Opaline motor neurons in the right pleural ganglion are similar in biophysical properties to the ink-release neural system, which has led authors to believe that the opaline gland is also involved in defensive behavior (Tritt and Byrne, 1980; reviewed in Johnson and Willows, 1999). The opaline gland is a variable anatomical trait in the genus *Aplysia* and especially within the subgenus *Varria.* Nevertheless, it has a uniform anatomical structure within all members of the subgenus *Aplysia* (Eales, 1960; Johnson and Willows, 1999). If, in the species of this subgenus, the ink gland has lost importance as a predator deterrent organ because of its inability to produce purple ink, then the opaline gland could play a critical role in defensive behavior. Therefore, the rigid anatomy of the opaline gland within the subgenus *Aplysia* could be explained by stronger selective constraints on this structure. In the subgenus *Varria*, selection on the opaline gland may be reduced, because all *Varria* species sequester purple ink in their vesicles. The relaxed selective pressure on the opaline gland could explain its anatomical plasticity in this subgenus.

In this case, the molecular phylogeny allowed us to identify the potential loss of

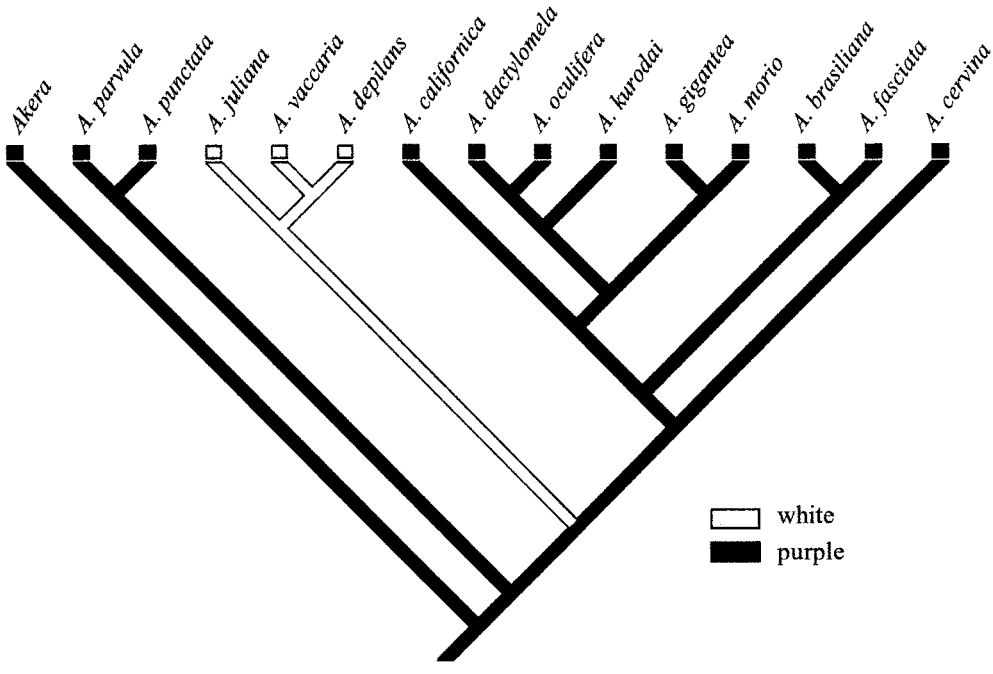

FIGURE 8. Inking behavior mapped onto the molecular phylogeny. Inking (the release of a purple secretion from the ink gland) appears to have been lost in the members of the subgenus *Aplysia* (data from Eales, 1960; Carefoot, 1987; T. Carefoot, T. Gosliner, P. M. Johnson, and S. Pennings, pers. comm.).

function of the ink gland in the subgenus *Aplysia* and to hypothesize the potential defensive role that the opaline gland may play in this group. The phylogeny could serve as a guide in selecting taxa for testing competing hypotheses concerning the function and control of the opaline gland as well as understanding the relationship between the neural pathways that control both the ink and opaline glands.

Evolution of Siphon and Gill Withdrawal

Siphon and gill withdrawal reflexes are one of the major targets of study in the neurobiology of *Aplysia* (Kupfermann and Kandel, 1969; Frost and Kandel, 1995). The neural circuit for these two reflexes is known to involve >100 neurons in the abdominal ganglion (Frost and Kandel, 1995). Most of the studies have been performed on *A. californica*, although recent studies have been published that compare different species and different genera (Wright et al., 1996; Wright, 1998; Kurokawa et al., 1998; Erixon et al., 1999). Kurokawa et al. (1998) studied siphon and gill withdrawal in three species (*A. californica*, *A kurodai*, and *A. juliana*), two from the subgenus *Varria* and one from the subgenus *Aplysia*, respectively. They determined that in all three species the neuron L7 of the abdominal ganglion acts both as a motor neuron and as an interneuron of the gill. This finding, however, applies only to the more derived sea hares; the question of functional homology remains to be determined for the basal taxa of the subgenus *Pruvotaplysia*. Collection of additional data for more primitive sea hares will allow for a better understanding of the evolution of this behavioral mechanism.

Conclusions

The molecular phylogeny presented here is intended to help address evolutionary hypotheses of neurophysiological, neuroethological, ecological, and taxonomic interest by providing a topology on which to map the evolution of particular traits. This phylogenetic framework should allow neurobiologists to make reasonable inferences concerning the ancestral condition, timing of evolution, and number of independent acquisitions of traits of interest. In particular, the demonstration of cases of independent acquisition of some behaviors will enable neurobiologists to address the relationship

between behaviors and their underlying neurological bases in phylogenetically independent comparisons.

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